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Comparison of Phosphatidylcholine Vesicle Properties Related to Geometrical Isomerism†

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ABSTRACT

Glycerophosphatidylcholine containing trans-unsaturated fatty acid residues was prepared by reaction of the corresponding naturally occurring cis lipid with photochemically generated thiyl radicals. This modified lipid was chosen as the simplest model for gaining some insights of the complex scenario of membrane formation, in connection with the role of lipid geometry and the predominance of cis lipids in eukaryotic cells. The critical aggregation concentration for the spontaneous formation of vesicles was determined for cis and trans isomers with *cis*-parinaric acid used as a fluorescent probe and it was found to be similar for both lipids. Vesicle dimensions were investigated by light scattering and electron microscopy, and the type of fatty acid residues influenced the vesicle diameter, with a decrease along the series cis > trans > saturated. Fluorescence measurement of dye release from trans and cis vesicles showed also a different permeability. A picture emerged of the geometrical isomer preference in cells as a process driven by natural selection during the life evolution of different organisms, both in terms of compartment dimensions and membrane functionality.

INTRODUCTION

Unsaturated fatty acid residues of glycerol-based phospholipids in eukaryotes generally have the naturally occurring cis double-bond geometry, which is strictly controlled during biosynthesis by the regiospecific and stereoselective enzymatic activity of desaturases (1,2). Membrane physical and functional properties, such as chain melting transition temperature (T_m), "fluidity" and permeability, have a fine balance, which is also regulated by the appropriate ratio between saturated and cis unsaturated fatty acids (FA).

Considering the variety of glycerophospholipids, which are the most abundant lipids in cell membranes, those containing fatty acid residues with the trans configuration of double bonds are not natural in eukaryotes. They have attracted attention because of their growing relevance in nutrition and health. The effect of trans fatty acid intake has been the subject of several investigations, which evidenced how trans isomers can be metabolized and become constituents of membrane phospholipids. Epidemiological studies have also linked this intake with health complications (3–7). In an effort to understand the potential harmful effects of trans phospholipids in membranes, some recent articles reinvestigated the different membrane properties induced by cis and trans geometries (8,9). Recently, it has also been shown that trans lipid geometry influences the affinity of membranes for cholesterol incorporation and the activity of eco-Ri protein (10). These works, together with previous data (11,12), contributed to a comprehensive picture of the lipid geometry strictly involved with the organization and functioning of cell membranes.

On the other hand, trans geometry of membrane phospholipids is natural for some bacteria and means survival. In fact, unsaturated fatty acid moieties are essential for a short-term adaptation response to increases in the ambient temperature or high concentrations of toxic substances (13,14). They are enzymatically converted from cis to trans isomers, without shifting the double-bond position. Analogous enzymatic pathways for the cis-trans isomerization in mammals are unknown.

We became interested in the biological meaning of trans lipid geometry (15,16) in the course of our studies on the thiyl radical-catalyzed cis-trans isomerization of phospholipids in model membranes (17–19). This process occurs by the addition of RS$^\bullet$ radicals to the double bond and subsequent β-fragmentation of the radical adduct, as shown in Fig. 1, wherein the equilibrium is shifted to the right toward the more stable geometrical trans isomer.

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Abbreviations: CAC, critical aggregation concentration; CF, 5-(and-6)-carboxyfluorescein; *cis*-PnA, *cis*-parinaric acid; DPPC, 1,2-dipalmitoyl phosphatidylcholine; DSC, differential scanning calorimetry; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUVET, large unilamellar vesicle by extrusion technique; MOPS, 3-(*N*-Morpholino)propanesulfonic acid; PEPC, 1-palmitoyl-2-elaidoyl phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine.

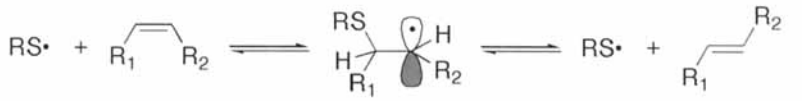


Figure 1. The isomerization process occurs via a thiyl radical addition to the double bond and subsequent β-elimination from the intermediate radical adduct.

The influence of kinetic and thermodynamic factors on the process was also determined (20).

Taking into account the chemical behavior in favor of trans structures and the biological predominance of cis isomers, we focused on the reason why only the cis geometry is present in eukaryotic unsaturated fatty acids. Curiously, this question has been omitted from the interesting debates of the fatty acid presence during the evolution of cell membranes and mankind (21–23). Indeed, the most used argument is that cis lipids are needed for an optimal cell membrane balance. However, it is not straightforward whether trans isomers have been *a priori* excluded from the eukaryotic cell membrane composition, or whether this is a result of life evolution and selection. The fact that some bacteria still use trans lipid geometry could favor the hypothesis of an evolutionary meaning.

An inspection of the literature reveals that relevant information on the influence of trans lipids is still lacking, in particular the critical aggregation concentration (CAC) to form spontaneously unilamellar vesicles and the resulting average dimensions. Dimensions are indeed relevant in prokaryotic and eukaryotic cells (24,25).

In the present work the photochemical preparation of a trans FA containing glycerophosphatidylcholine is described, and new insights on spontaneous vesicle formation, the CAC value, vesicle permeability and dimensions and the behavior of the so-called "matrix" effect, also were determined under physiological conditions. Fluorescence and light scattering techniques, together with electron microscopy, were used with cis and trans vesicle suspensions. An initial scenario is obtained indicating the directions for further interdisciplinary work, with a focus on the meaning of the unsaturated lipid geometry in the biological environment.

MATERIALS AND METHODS

2-mercaptoethanol, bicine[*N,N*-bis-(2-hydroxyethyl)glycine], MOPS [3-(*N*-morpholino) propanesulfonic acid] and DPPC (1,2-dipalmitoyl phosphatidylcholine) were commercially available from Sigma-Aldrich (Milan, Italy) and used without further purification. Tris (tris-(hydroxymethyl)-aminomethane) was from Merck (Darmstadt, Germany). POPC (1-palmitoyl-2-oleoyl phosphatidylcholine) was from Chemi SpA (Cinisello Balsamo, Milan, Italy). Isopropanol (i-PrOH), absolute ethanol, chloroform and methanol were purchased from Merck or Sigma-Aldrich (HPLC grade). *cis*-parinaric acid (9cis,11trans,13trans,15cis-octadecatetraenoic acid) and 5-(and-6)-carboxyfluorescein (CF) were obtained from Molecular Probes, Inc. (Eugene, OR). Chromatography was performed on Florisil® (TLC grade, Sigma-Aldrich) by using the eluent as specified in the protocols and analytical thin layer chromatography (TLC) on Merck silica gel 60 plates. Lecithin analysis for cis/trans isomer content was performed by transesterification to the corresponding fatty acid methyl esters (FAME) with 0.5 *M* KOH/MeOH for 10 min at room temperature (26) and GC analysis as previously reported (17).

POPC isomerization by photochemical reaction. A quartz photochemical reactor equipped with a 5.5 W low-pressure mercury lamp was used, and the temperature was maintained constant by means of a thermostat bath. A solution of POPC (60 mg; 0.079 mmol) in isopropanol (5.2 mL) was placed in the reactor and bubbled with argon for 20 min. Then, 2-mercaptoethanol (3 mg; 0.039 mmol) was added and the solution was irradiated for 30 min at 22°C. After this time, the solution was transferred to a flask and evaporated under vacuum to afford a crude reaction mixture, which was dissolved in chloroform and purified on a Florisil column. After washing with a 80:20 chloroform:methanol mixture, the lecithin was collected in one fraction by elution with a (20:10:0.4:0.2) chloroform:methanol:water:25% aq. NH_4OH mixture. The purity of the material was checked by TLC on silica gel, with the use of the above-specified eluent (R_f = 0.51). The purified material corresponded to pure lecithin (59 mg; 0.077 mmol; 98% yield), which was analyzed by GC for the trans isomer content and resulted to be composed of 83:17 trans:cis isomer (PEPC-83). The synthesis could be repeated on a 10-fold scale with reproducible results.

Phase transition temperature measurement. The measurements were performed by differential scanning calorimetry (DSC) with the use of a TAC7/DX DSC 7 system (Perkin Elmer, Boston, MA). The sample was prepared in a glass tube by dispersing appropriate amounts of lipid (28.5 mg/mL) in 0.22 *M* bicine buffer, pH 8.5. An aliquot of the sample (20 mg) was put into aluminum DSC pans and placed in the DSC instrument. The sample was cooled down slowly (10°C/min) to 0°C and kept at this temperature for 10 min. The DSC trace was then obtained by slowly heating the sample (10°C/min) to a final temperature of 60°C. The phase transition temperature was determined by extrapolating the slope of the onset of the transition to the baseline. Transition enthalpy was calculated from the DSC peak area. Cooling and heating processes were repeated for the sample, and it was confirmed that the DSC traces were reproducible.

Vesicle preparation by the injection method and light scattering measurements. POPC, PEPC-83 or DPPC was first dissolved in methanol at room temperature. Aqueous lipid suspensions were then prepared by injecting the methanolic solution through a Hamilton syringe (Bonaduz, Switzerland) into 10 mL of a slowly stirred buffer solution (27,28). Two buffer solutions were used: 0.2 *M* bicine at pH 8.5, or 0.01 *M* potassium phosphate buffer at pH 7.4. In both cases these were filtered through a 0.2-μm filter (Corning, Inc., Acton, MA) in order to avoid dust-particle interference. Vesicle suspensions with a final lipid concentration in the range 0.004–0.16 m*M* were prepared for the measurements. During the injection, the temperature was kept at 30 ± 1°C for both POPC and PEPC-83, and at 50°C when DPPC was used. A 2.5 mL amount of the phospholipid suspension was transferred to a quartz cell, and measurements were done by a Zetasizer mod. 3000 (Malvern Instruments Ltd., Malvern, UK), with a 90° scattering angle. Temperature was set at 30 or 50°C, and the measurement started after 5 min to allow for temperature equilibration.

Fluorescence measurements and vesicle formation. Fluorescence measurements were recorded on a Perkin Elmer Luminescence Spectrometer LS 50 B. Lipid suspensions were kept at 35°C, and for DPPC suspensions the temperature was set at 50°C during the preparation, incubation and measurements. Suspensions were prepared at 0.001 *M* lipid concentration by the injection methodology, as previously described, with the two previously described buffers. From this suspension, the other samples were obtained by serial dilution. The final phospholipid concentration varied between 1.25×10^{-8} and 5×10^{-4} *M*. Fresh *cis*-parinaric acid (*cis*-PnA) solutions were prepared each working day by dissolving a small amount of crystals in absolute ethanol previously degassed with argon (29,30). Because of large insoluble particles, *cis*-PnA stock solution was then filtered, and probe concentration was determined by measuring UV absorption at λ_{max} = 304.2 nm (ε = 78 000 M^{-1} cm^{-1}). If necessary, *cis*-PnA stock solution was diluted in order to have $Abs(\lambda_{max}) < 1$.

The lipid probe solution was added to the vesicle suspension to reach the final concentration of 1 μ*M*. Samples were then left 3 h at the defined temperature before measurements were taken. The spectrofluorimetric detection was performed at 415 nm upon excitation at 305 nm.

Determination of the "matrix" effect. Addition of oleate micelle solution to pre-formed vesicles. Oleate stock solution (22 m*M*) was prepared by solubilizing a certain amount of sodium oleate (0.067 g; 0.022 mmol) into ultrafiltered water (1 mL). A 200 μL amount of this micellar solution (pH 10.5) was injected with a Hamilton syringe directly into a quartz cell containing 2 mL of preformed PEPC-83 vesicle suspension in 0.2 *M* bicine buffer, pH 8.5, as previously described. After the injection, the cell was gently hand shaken (three times), then inserted into the cell holder.

Electron microscopy measurements of vesicle dimension. A 10 μL amount of vesicle suspension (1 mg PC/mL) prepared as previously described was adsorbed to Formvar and carbon-coated 300-mesh nickel

grids (Canemco Inc., Canton de Gore, Canada) for 3 min and negatively stained with 2% phosphotungstic acid pH 7.4 (for 2 min). After removal of the excess of stain, the specimen was air dried and observed with a Zeiss EM 109 electron microscope (Jena, Germany).

Permeability measurements. Leakage of carboxyfluorescein from vesicles of different composition. Multilamellar vesicles (MLV) containing 5-(and-6)-carboxyfluorescein (CF) were prepared from 3 mg POPC or PEPC-83 in chloroform. A thin lipid film was generated in a round-bottom tube by evaporation of the solvent under a gentle N_2 stream followed by rehydration of lipids at room temperature in 0.5 mL of buffer (0.5 *M* MOPS, pH 6, and NaOH) containing CF to reach a final 53 m*M* concentration of the dye. Then, phospholipids were dispersed by vortex stirring for 10 min and vesicles were annealed for one night in the dark at 37°C. Unilamellar vesicles (LUVET) were obtained by extruding 0.5 mL of the MLV suspension 21 times through 100-nm pore polycarbonate membranes (Nucleopore, Pleasanton, CA) with LiposoFast (Avestin, Ottawa, Ontario, Canada). The untrapped CF was removed by passing the unilamellar vesicles through Sephadex G-25 columns (Pharmacia, Uppsala, Sweden) with 50 m*M* Tris-HCl, 100 m*M* NaCl, pH 7.4, as eluent. Vesicles emerged in the void volume, whereas free dye was retarded by the gel. Each preparation was kept in the dark until use. To measure CF release, vesicles were diluted in 3 mL of Tris-HCl buffer to yield a final CF concentration of 50–100 n*M*. The probe concentration was determined spectrophotometrically, assuming a molar extinction coefficient of 72 000 M^{-1} cm^{-1} at 492 nm (31). Fluorescence measurements were performed at 37°C in a Jasco FP-777 spectrofluorometer (JASCO Ltd., Great Dunmaw, UK) at 520 nm upon excitation at 470 nm, and the samples were maintained under continuous stirring.

RESULTS AND DISCUSSION

Photochemical generation of thiyl radicals and lipid isomerization

The UV photolysis (250–260 nm) of alkanethiols has been studied in some detail in both gas and liquid phase, and the reaction products are consistent with Eqs. 1–3 (32,33)

$$RSH + h\nu \rightarrow RS^{\bullet} + H^{\bullet} \quad (1)$$

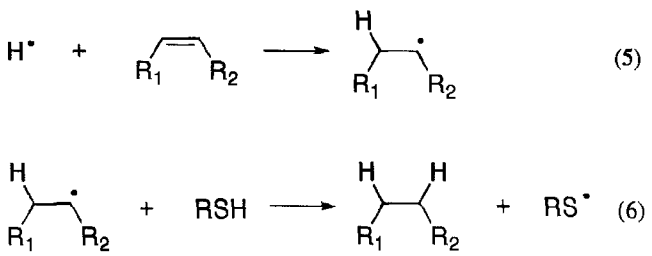
$$H^{\bullet} + RSH \rightarrow H_2 + RS^{\bullet} \quad (2)$$

$$2RS^{\bullet} \rightarrow RSSR \quad (3)$$

In our previous studies (16–20), amphiphilic $HOCH_2CH_2SH$ was often used without any concern about the partition of thiol between hydrophobic and hydrophilic regions (34). However, $H^{\bullet}$ atoms react with $HOCH_2CH_2SH$ by two distinct paths. Apart from the hydrogen abstraction that occurs with a $k_2 = 1.4 \times 10^9$ M^{-1} s^{-1}, a homolytic substitution at sulfur is also effective, as shown in Eq. 4 ($k_4 = 3.3 \times 10^8$ M^{-1} s^{-1}), the preference being 4:1 in favor of hydrogen abstraction (35).

$$H^{\bullet} + HOCH_2CH_2SH \rightarrow HOCH_2CH_2^{\bullet} + H_2S \quad (4)$$

When the photolysis of 0.15 *M* methyl oleate and 0.1 *M* $HOCH_2CH_2SH$ was carried out in deareated *tert*-butanol (t-BuOH), to our surprise large amounts (up to 20%) of methyl stearate (saturated) were found together with the expected elaidate, the trans isomer. Under these conditions, $H^{\bullet}$ atoms add to the double bond (Eq. 5) and the resulting carbon-centered radical reacts with RSH (Eq. 6) to give the hydrogenated olefin. Indeed, $H^{\bullet}$ atoms are known to add to 1,2-dialkyl substituted olefins very fast $k_5 = 3 \times 10^9$ M^{-1} s^{-1} (35).



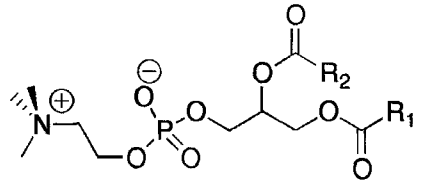
However, a cleaner photochemical method for the induction of the desired isomerization without hydrogenation of double bonds was obtained by replacing t-BuOH with i-PrOH as the solvent. Under this condition, $H^{\bullet}$ atoms are completely quenched by the solvent ($k_7 = 5.3 \times 10^7$ M^{-1} s^{-1}) and the resulting alkyl radical reacts with the thiol, as shown in Eqs. 7 and 8

$$H^{\bullet} + (CH_3)_2CHOH \rightarrow H_2 + (CH_3)_2C^{\bullet}OH \quad (7)$$

$$(CH_3)_2C^{\bullet}OH + RSH \rightleftharpoons (CH_3)_2CHOH + RS^{\bullet} \quad (8)$$

It is worth pointing out that the reaction in Eq. 8 is reversible (36,37). Forward and reverse rate constants for the reaction of $(CH_3)_2C^{\bullet}OH$ radical with penicillamine are $k_8 = 1.2 \times$ and $k_{-8} = 1.2 \times 10^4$ M^{-1} s^{-1}, respectively, which correspond to an equilibrium constant of $K = 1 \times 10^4$. Under our experimental conditions, that is, [i-PrOH] = 13.06 *M* and [$HOCH_2CH_2SH$] = 0.007 *M*, the equilibrium is still shifted to the right but the forward reaction is only 5–6 times faster than the reverse reaction.

The simplest model for investigating cis and trans differences consisted of 1-palmitoyl-2-oleoyl L-α-phosphatidylcholine (POPC, **1**), having the monounsaturated chain of oleic acid (9cis-C18:1), and its geometrical isomerization product. In fact, the thiyl radical catalyzed process converts oleic acid to the corresponding trans fatty acid, elaidic acid (9trans-C18:1), in an isomeric abundance that is regulated by the thermodynamic equilibrium of Fig. 1 (17,20). The final product is a trans FA containing phosphatidylcholine, which is composed by 83% of 1-palmitoyl-2-elaidoyl L-α-phosphatidylcholine (PEPC, **2**) and 17% of POPC. We denoted this mixture as PEPC-83. A degassed i-PrOH solution of POPC was added with 2-mercaptoethanol. This solution was photolyzed at 22°C with a 5.5 W low-pressure Hg lamp. After about 30 min the corresponding product PEPC-83 was obtained in a quantitative yield. After purification, the trans content was established by conversion to the corresponding FAME (26) followed by GC analysis (17).



1 (POPC): R_1 =palmitoyl, R_2 =oleoyl,
2 (PEPC): R_1 =palmitoyl, R_2 = elaidoyl

Phase transition temperature measurements. The chain melting transition temperature T_m represents a relevant physical characteristic for lipids. For a certain lipid or a lipid mixture it is the temperature of the transition between the liquid crystalline and gel phases. Pure PEPC is reported to have phase transition temperature of 35°C, whereas –3°C is the T_m of POPC (38). With the use of the differential scanning calorimeter technique (DSC), PEPC-83 transition temperature was determined to be 22.7°C.

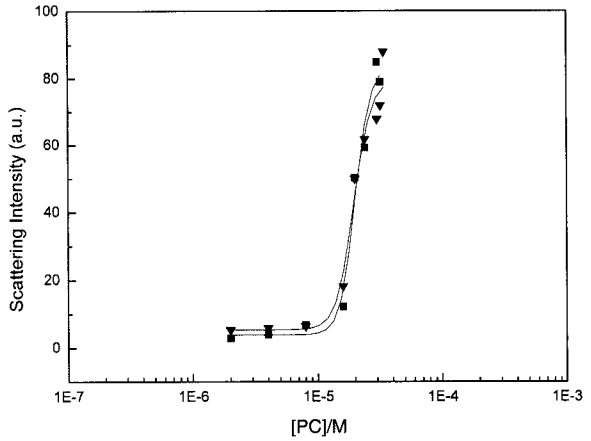


Figure 2. Light scattering intensities of POPC (squares) and PEPC-83 (triangles) in 0.01 *M* phosphate buffer, pH 7.4; scattering angle 90° at 30°C.

The spontaneous vesicle formation

With PEPC-83 the formation of unilamellar vesicles by spontaneous aggregation was addressed, in comparison with the corresponding natural cis lipid POPC, with a particular attention to biologically related conditions. The CAC of lipids provides information of the minimum lipid concentration needed to form a vesicle, and it ranges from micromolar to picomolar concentrations in aqueous solution (38,39). The values for cis phospholipids are reported in different conditions and some data are very old. Recent data of 8:2 POPC:phosphatidylserine mixture vesicles are reported in 10 m*M* HEPES buffer at pH 7.2, with the addition of 1 *M* lithium hydroxide, and the CAC value is 1×10^{-6} *M* (40). For trans phospholipids, no data are available under more biologically related conditions. It is known that carbon atom chain length influences the CAC values (38), but in case of geometrical isomers, only the configuration of the double bond changes. On the other hand, the cis and trans aggregation modes could be different, due to the different structural features of the two lipids. In fact, cis double bonds confer a kink in the carbon atom chain of the fatty acid residue, so that the spatial width of cis lipids results to be larger than that of the corresponding saturated or trans structures (9,38). The CAC values were determined by two methodologies, namely, light scattering and spectrofluorimetric measurements with a fluorescent probe inserted in the bilayer.

Light scattering measurements. CAC measurements of POPC and PEPC-83 vesicles were performed at the temperature of 30°C, which is above both lipid phase transition temperatures. Vesicle suspensions were prepared by the injection method, that is, by injecting lipid methanolic solution in the buffer solution. In this way, unilamellar vesicles are formed (27,28). Bicine buffer, pH 8.5 or phosphate buffer, pH 7.4, were used and no significant differences between the two buffer systems were observed. Under varying lipid concentrations, a scattering intensity is expected only when vesicles are present. In Fig. 2 the scattering intensities are shown for POPC and PEPC-83 at various concentrations. The CAC values resulted from the sharp change of scattering intensity and the changes occurred at a similar concentration for the two lipids, that is, at 1.6×10^{-5} *M* for POPC and 1.3×10^{-5} *M* for PEPC-83.

Because CAC values were similar, the other parameter to be determined was the vesicle diameter given by cis and trans geometries. With the use of the light-scattering apparatus, this measurement was carried out with a PEPC-83 molarity of 0.03 *M*, a lipid concentration where the vesicles are present (cf. Fig. 2). PEPC-83 measurement also paralleled that of a saturated lipid, such as 1,2-dipalmitoyl phosphatidylcholine (DPPC), and the cis lipid, POPC. The comparison was made at 50°C, a temperature that is above the phase transition temperatures of all three phospholipids. Interestingly, trans phospholipid vesicle diameter was found to be 25% smaller than that of cis vesicles. Table 1 reports the values as the average of three different experiments. The polar head is identical for the three phospholipids, so the influence of fatty acid residues can be appreciated and vesicle diameter increases in the order saturated < trans < cis. The substantial diameter difference observed between saturated and trans lipids is remarkable, as trans vesicles are 60% larger than saturated ones.

Table 1. Dimensions of vesicles formed spontaneously in 0.01 *M* phosphate buffer, pH 7.4, at 50°C, as determined by light scattering measurements (scattering angle 90°)

Phospholipid*	Diameter (nm)†
POPC	97 ± 10
PEPC-83	72 ± 8
DPPC	45 ± 8

* Vesicles are obtained by methanolic injection to reach a 0.03 *M* lipid concentration in buffer.

† Values are reported as mean values ± SD obtained from three different experiments. Each experiment was carried out by setting $n = 5$ repetitions on the instrument.

Evaluation of cis and trans vesicle properties

Fluorescence measurements. The properties of vesicles of different compositions can be studied by means of a fluorescent probe inserted within the bilayer. We chose *cis*-parinaric acid as the probe, because this molecule is known to be fluorescent only when it is within the lipid bilayer (29,30). Suspensions of POPC and PEPC-83 were prepared by the injection method, and then lower PC concentrations were obtained by diluting the former suspension. The buffers used were 0.2 *M* bicine, pH 8.5 or 0.01 *M* potassium phosphate, pH 7.4, and the temperature was set at 35°C. To each of these suspensions, the same amount of the probe was added to reach a final 1 μ*M* concentration, and the fluorescence emission was monitored at 415 nm upon excitation at 305 nm. The results were independent of the buffers used and similar for cis and trans isomers. Figure 3 shows a representative behavior of *cis*-PnA fluorescence emission as a function of POPC or PEPC-83 concentrations in phosphate buffer (Fig. 3, A and B, respectively). It can be seen that fluorescence disappeared below a certain lipid concentration, evidently due to the lack of a lipid environment for probe insertion. The smallest concentration for observation of fluorescence was the same for POPC and PEPC-83, in the range of $4–5 \times 10^{-6}$ *M*. This analysis provided a complementary evaluation of the vesicle formation process in condition of progressive dilution. In other words, the preformed unilamellar vesicles are diluted until they no longer reach the minimum lipid concentration to remain as aggregates. In absolute values, this latter methodology gave different values from the previous one with light scattering intensity. This could be due to interferences from dust particles during measurement, which are known to affect the sensitivity. An in-depth evaluation of the methodologies, however, is beyond the scope of the present work. Both results confirmed that the

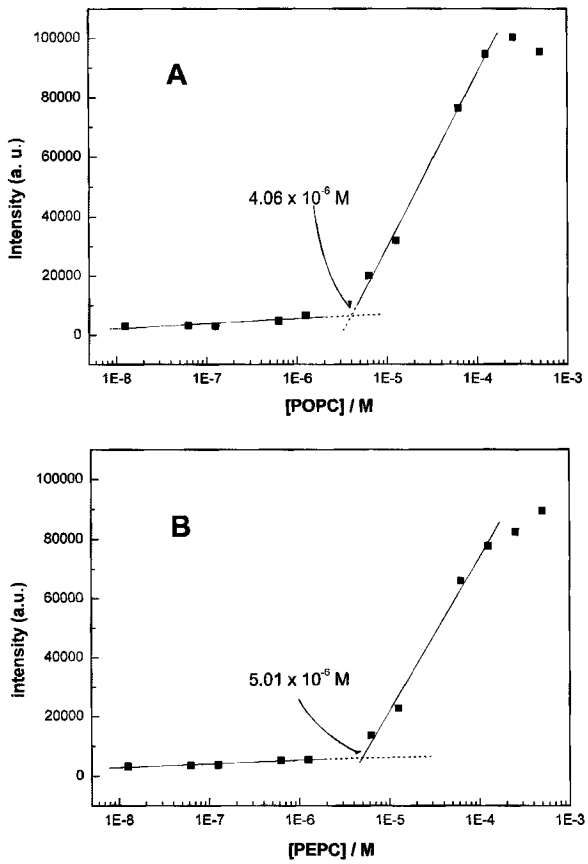


Figure 3. *cis*-Parinaric acid fluorescence emission in POPC (A) and PEPC-83 (B) vesicles. Measurements were carried out at 35°C in 0.01 *M* phosphate buffer, pH 7.4 (λ_{exc} = 305 nm; λ_{em} = 415 nm).

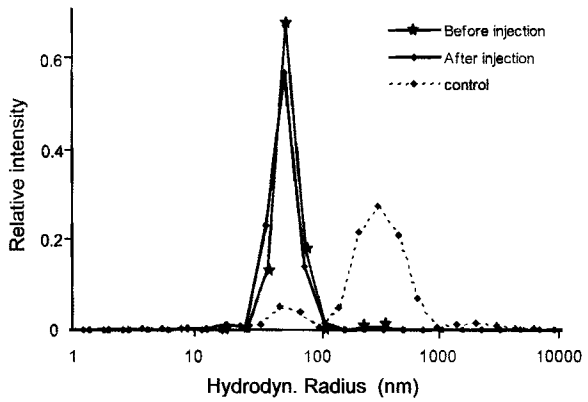


Figure 4. Intensity-weighted size distribution calculated from DLS by Contin analysis (scattering angle 90°, λ = 633 nm, temperature 30°C). The first trace (monomodal distribution) is related to PEPC vesicle dimension, and the second trace to the addition of sodium oleate micelle solution injected into the PEPC suspension (1:1 ratio; final lipid concentration 4 m*M*). The dashed line (bimodal distribution) is related to the blank experiment, that is, the injection of sodium oleate micelle suspension in the buffer.

aggregation modes of cis and trans lipids are not significantly different. Therefore, it can be concluded that the process of vesicle formation is independent from the geometry of the lipid molecule.

The "matrix effect." The phenomenon of matrix effect was reported some years ago after the observation of the influence of preformed vesicles on the organization of newly formed micelles or vesicles (41). This is a complex effect given by preformed vesicles, which influence the further formation of vesicles or micelles from an added lipid, in terms of the size distribution and kinetics. For example, it was ascertained for preformed POPC vesicles that added oleate micelles aggregate much faster, and the final distributions appeared very close to the initial ones, demonstrating the effective matrix role of POPC vesicles. The ratio between POPC and the added component is also very important. The matrix effect shows that there is an important interaction between the two systems (POPC vesicles and oleate micelles) and favors the "division" hypothesis, based on the self-replication of the vesicles, which keeps the sizes as the initial values (42). The dynamic light scattering technique represents a good approach for understanding the behavior of the system and its dimensional changes. In analogy with previous studies, 0.2 *M* bicine buffer, pH 8.5, was used as the medium where a 2.2 m*M* PEPC-83 suspension was prepared at a temperature of 30°C. A 22 m*M* sodium oleate micelle solution in methanol was injected in the preformed PEPC-83 vesicle suspension, in order to reach a final total lipid concentration of 4 m*M*. The size distribution before and after the oleate micelle injection was evaluated by light scattering, and the results are shown in Fig. 4.

The influence of PEPC liposomes on the final size distributions is clear, because there is no relevant change of the monomodal size distribution before and after injection. For comparison the sole oleate micelle suspension was also injected in the buffer and a bimodal size distribution was found (Fig. 4, dashed line). The "matrix effect" was thus confirmed for the trans unsaturated vesicles as preexisting vesicles; therefore, if trans components are first present, they can drive the size distribution of vesicles subsequently formed by addition of other lipid components.

Permeability measurements by the leakage of carboxyfluorescein (CF) from vesicles. The permeability of vesicles formed by POPC and PEPC-83 was evaluated by measuring the spontaneous release of the hydrophilic and polar fluorescent dye CF, encapsulated in the vesicles at a self-quenching concentration (31). Encapsulation was carried out as already described (43). Leakage into the surrounding medium was monitored as the increase of fluorescence intensity due to the dilution of the water-soluble probe. The leakage was examined from unilamellar vesicles obtained by extrusion through a 100 nm polycarbonate filter (44) at 37°C, which is well above the phase transition temperatures of the two lipids. Figure 5 shows the time-dependent increase of fluorescence due to the release of the dye from POPC and PEPC-83 vesicles. Enhancement of the fluorescence intensity was slow in the case of trans-PC vesicles, whereas the release from vesicles composed of cis-unsaturated phospholipids occurred at higher rates.

This behavior is due to the different packing of trans-acyl chains that are more ordered than cis-acyl chains, and it is known that ordered lipid structure decreases solute permeability (9,11,43). Thus, the experiments confirmed that functional properties of trans-model membranes, such as permeability, are different from the natural cis membranes. It is worth recalling that an even larger change in the barrier properties occurs in the presence of a saturated bilayer, and the regulation of fluidity and permeability of membranes results from a fine balance of several structural contributions, including lipid polar heads and hydrocarbon tails.

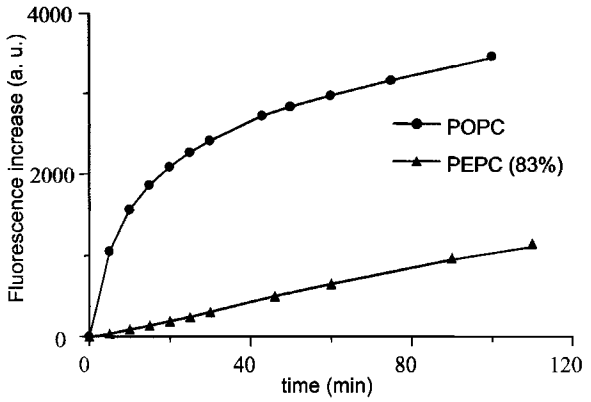


Figure 5. Time course of CF release from unilamellar vesicles composed of either POPC or PEPC-83 incubated in 50 m*M* Tris-HCl, 100 m*M* NaCl, pH 7.4, at 37°C.

Based on the overall knowledge on the different contributions given by saturated, cis and trans unsaturated fatty acid residues, it can be concluded that a balance of the functional properties of membranes could have been achieved by mixing these three components. Under this point of view, the exclusion of trans structures from the eukaryotic membranes is not straightforward.

Electron microscopy measurements. A relevant difference among saturated, cis and trans lipid structures, as emerged in the light scattering analyses, was the vesicle diameter. This feature was also investigated by using electron microscopy. After preparation of POPC and PEPC-83 vesicles in buffer, pH 7.4, by the injection method, as previously described, suspensions were used for examination with electron microscope after negative staining with 2% phosphotungstic acid. Figure 6 shows representative pictures of cis and trans vesicles, which are mostly unilamellar with a regular size, as expected from the methodology (27,28). By evaluation of an average number of vesicles (n = 100), the trans vesicle diameter was found to be 101 ± 7 nm, whereas cis vesicle diameter was 127 ± 8.9 nm. Thus, cis and trans vesicle diameter differs by ca. 25% ($P < 0.0001$), as shown above by light scattering analysis (*vide supra*).

CONCLUSIONS

Cis-trans isomerization by photochemically generated thiyl radicals provides a general and easy methodology to prepare trans-FA-containing phospholipids. In this article PEPC-83 was prepared, a phosphatidylcholine containing 83% of a trans fatty acid isomer such as elaidic acid, and new data were obtained related to the influence of geometrical trans lipid isomers in model membranes.

Chain melting transition temperature T_m, CAC value and vesicle dimension are here reported for the first time for a trans-FA-containing lipid. Comparison of CAC values with the corresponding cis lipid under identical conditions revealed that the two geometrical lipid isomers aggregate at almost the same concentration. On the other hand, the diameter of the cis vesicle was found to be 25% larger than the corresponding trans-FA-containing vesicle. The type of fatty acid residues influence the vesicle dimensions, which decrease along the series cis > trans > saturated. Furthermore, the influence of the trans geometry on membrane permeability was also assayed.

What is the contribution of the present work to the original question of the *a priori* exclusion of a trans lipid from the eukaryotic cell membrane composition? From our data, it seems to be traced back to effects of a natural selection obtained during cell evolution, both in terms of compartment dimensions and functions. This motivates further work on models where trans fatty acids are mixed at different concentrations with the other naturally occurring lipids, to investigate the minimum trans contents influencing vesicle characteristics. The presented data, together with the known properties of cis and trans isomers, give a contribution to the complex scenario of membrane formation, for a full understanding of the role of lipid geometry in living organisms.

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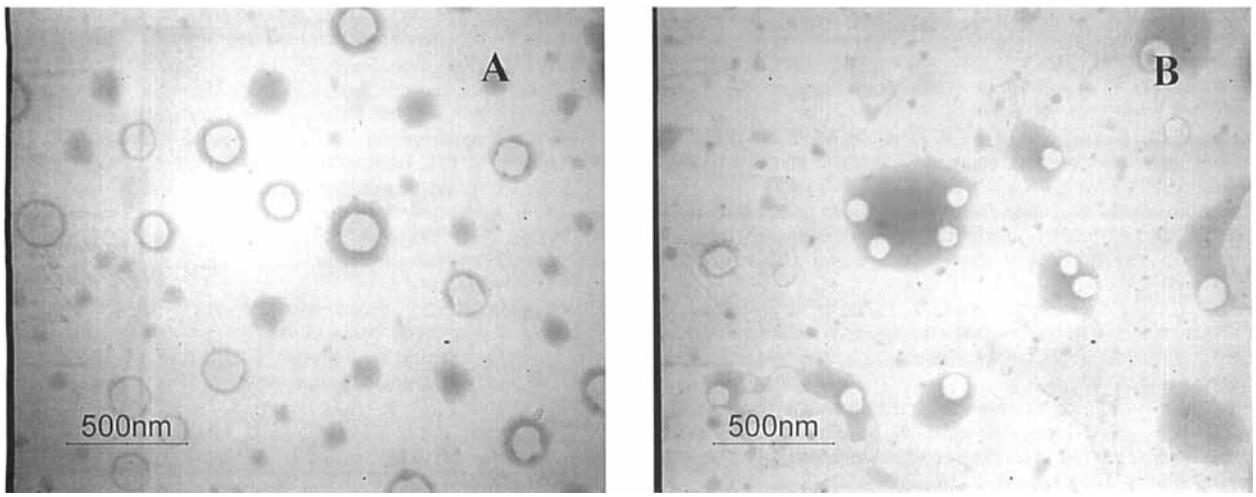

Figure 6. Micrography of cis (A) and trans (B) vesicles formed by the injection method.

REFERENCES

1. Vance, D. E. and J. E. Vance, editors (2002) *Biochemistry of lipids, lipoproteins and membranes* 4th ed. Elsevier, Amsterdam, The Netherlands.
2. Fox, B. G., K. S. Lyle and C. E. Rogge (2004) Reactions of the diiron enzyme stearoyl-acyl carrier protein desaturase. *Acc. Chem. Res.* **37**, 421–429.
3. Sébédio J. L., V. S. H. Fermunt, J. M. Chardigny, B. Beaufrere, R. P. Mensink, R. A. Armstrong, W. W. Christie, J. Niemelä, G. Hènon and R. A. Riemersma (2000) The effect of dietary trans α-linolenic acid on plasma lipids and platelet fatty acid composition: the TransLinE study. *Eur. J. Clin. Nutr.* **54**, 104–113.
4. van de Vijver, L. P. L., A. F. M. Kardinaal, C. Couet, A. Aro, A. Kafatos, L. Steingrimdottir, J. A. Amorim Cruz, O. Moreiras, W. Becker, J. M. M. van Amelsvoort, S. Vidal-Jessel, I. Salminen, J. Moschandreas, N. Sigfusson, I. Martins, A. Carbajal, A. Ytterfors and G. van Poppel (2000) Association between trans fatty acid intake and cardiovascular risk factors in Europe: the TRANSFAIR study. *Eur. J. Clin. Nutr.* **54**, 126–135.
5. Sébédio, J. L. and W. W. Christie, editors (1998) *Trans Fatty Acids in Human Nutrition.* The Oily Press, Dundee, UK.
6. Alam, S. Q., Y. F. Ren and B. S. Alam (1989) Effect of dietary trans fatty acids on some membrane-associated enzymes and receptors in rat heart. *Lipids* **24**, 39–44.
7. Ibrahim, S. A. and S. A. Ghafoorunissa (2001) Influence of dietary partially hydrogenated fat high in trans fatty acids on lipid composition and function of intestinal brush border membrane in rats. *J. Nutr. Biochem.* **12**, 116–120.
8. Sargis R. M. and P. V. Subbaiah (2003) Trans unsaturated fatty acids are less oxidizable than cis unsaturated fatty acids and protect endogenous lipids from oxidation in lipoproteins and lipid bilayers. *Biochemistry* **42**, 11533–11543.
9. Roach C., S. E. Feller, J. A. Ward, S. Raza Shaikh, M. Zerouga and W. Stillwell (2004) Comparison of cis and trans fatty acid containing phosphatidylcholines on membrane properties. *Biochemistry* **43**, 6344–6351.
10. Niu, S. L., D. C. Mitchell and B. J. Litman (2005) Trans fatty acid derived phospholipids show increased membrane cholesterol and reduced receptor activation as compared to their cis analogs. *Biochemistry* **44**, 4458–4465.
11. Siminovitch, D. J., P. T. T. Wong and H. H. Mantsch (1987) Effects of cis and trans unsaturation on the structure of phospholipids bilayers: a high-pressure infrared spectroscopic study. *Biochemistry* **26**, 3277–3278.
12. Helmkamp, G. M. Jr. (1980) Effects of phospholipid fatty acid composition and membrane fluidity on the activity of bovine brain phospholipid exchange protein. *Biochemistry* **19**, 2050–2056.
13. Heipieper H. J., F. Meinhardt and A. Segura (2003) The cis-trans isomerase of unsaturated fatty acids in Pseudomonas and Vibrio: biochemistry, molecular biology and physiological function of a unique stress adaptive mechanism. *FEMS Microbiol. Lett.* **229**, 1–7.
14. Cronan J. E. Jr. (2002) Phospholipids modification in bacteria. *Curr. Opin. Microbiol.* **5**, 202–205.
15. Chatgilialoglu, C. and C. Ferreri (2005) Trans lipids: the free radical path. *Acc. Chem. Res.* **38**, 441–448.
16. Ferreri, C., S. Kratzsch, O. Brede, B. Marciniak and C. Chatgilialoglu (2005) Trans lipid formation induced by thiols in human monocytic leukemia cells. *Free Radical Biol. Med.* **38**, 1180–1187.
17. Chatgilialoglu C., C. Ferreri, M. Ballestri, Q. G. Mulazzani and L. Landi (2000) Cis-trans isomerization of monounsaturated fatty acid residues in phospholipids by thiyl radicals. *J. Am. Chem. Soc.* **122**, 4593–4601.
18. Ferreri, C., C. Costantino, L. Perrotta, L. Landi, Q. G. Mulazzani and C. Chatgilialoglu (2001) Cis-trans isomerization of polyunsaturated fatty acid residues in phospholipids catalyzed by thiyl radicals. *J. Am. Chem. Soc.* **123**, 4459–4468.
19. Ferreri, C., A. Samadi, F. Sassatelli, L. Landi and C. Chatgilialoglu (2004) Regioselective cis-trans isomerization of arachidonic double bonds by thiyl radicals: the influence of phospholipid supramolecular organization. *J. Am. Chem. Soc.* **126**, 1063–1072.
20. Chatgilialoglu, C., A. Altieri and H. Fischer (2002) The kinetic of thiyl-radical induced reactions of monounsaturated fatty acid esters. *J. Am. Chem. Soc.* **124**, 12816–12823.
21. Deamer, D., J. P. Dworkin, S. A. Sandford, M. P. Bernstein and L. J. Allamandola (2002) The first cell membranes. *Astrobiology* **2**, 371–381.
22. Segrè, D., D. Ben-eli, D. W. Deamer and D. Lancet (2001) The lipid world. *Origins Life Evol. B* **31**, 119–145.
23. Ackman, R. G. (1997) Has evolution and long-term coexistence adapted us to cope with trans fatty acids? *J. Food Lipids* **4**, 295–318.
24. Black, J. G. (1996) *Microbiology, Principles and Applications*, 3rd ed., pp. 75–78. Prentice Hall, Upper Saddle River, NJ.
25. Prescott, L. M., J. P. Harley and D. A. Klein (1996) *Microbiology*, 3rd ed., pp. 37–41. Wm. C. Brown Publishers, Dubuque, IA.
26. Kramer, J. K. C., V. Fellner, M. E. R. Dugan, F. D. Sauer, M. M. Mossoba and M. P. Yurawecz (1997) Evaluating acid and base catalysis in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. *Lipids* **32**, 1219–1228.
27. Domazou, A. S. and P. L. Luisi (2002) Size distribution of spontaneously formed liposomes by the alcohol injection method. *J. Liposome Res.* **12**, 205–220.
28. Batzri, S. and E. D. Korn (1973) Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta* **298**, 1015–1019.
29. Sklar L. A., B. S. Hudson and R. D. Simoni (1977) Conjugated polyene fatty acids as fluorescent probes: synthetic phospholipid membrane studies. *Biochemistry* **16**, 819–828.
30. Morgan, C. G., B. Hudson and P. K. Wolber (1980) Photochemical dimerization of parinaric acid in lipid bilayers. *Proc. Natl. Acad. Sci. USA* **77**, 26–30.
31. Barbet, J., P. Machy, A. Truneh and L. D. Leserman (1984) Weak acid-induced release of liposome-encapsulated carboxyfluorescein. *Biochim. Biophys. Acta* **772**, 347–356.
32. Calvert, J. G. and J. N. Pitts Jr. (1965) *Photochemistry*, pp. 488–492. Wiley, New York.
33. Knight, A. R. (1974) Photochemistry of thiols. In *The Chemistry of Thiol Group* Part 1. (Edited by S. Patai), pp. 455–479. Wiley, London.
34. Newton, G. L., J. A. Aguilera, T. Kim, J. F. Ward and R. C. Fahey (1996) Transport of aminothiol radioprotectors into mammalian cells: passive diffusion versus mediated uptake. *Radiat. Res.* **146**, 206–215.
35. Ross, A. B., W. G. Mallard, W. P. Helman, G. V. Buxton, R. E. Huie and P. Neta (1998) *NDRL-NIST Solution Kinetic Database—Ver. 3*, Notre Dame Radiation Laboratory, Notre Dame, IN and NIST Standard Reference Data, Gaithersburg, MD.
36. Schöneich, C., M. Bonifacic and K. D. Asmus (1989) Reversible H-atom abstraction from alcohols by thiyl radicals. Determination of absolute rate constants by pulse radiolysis. *Free Rad. Res. Commun.* **6**, 393–405.
37. Pogocki, D. and C. Schöneich (2001) Thiyl radicals abstract hydrogen atoms from carbohydrates: reactivity and selectivity. *Free Radical Biol. Med.* **31**, 98–107.
38. Cevc, G. editor (1993) *Phospholipid Handbook.* Marcel Dekker, New York.
39. Lide, D. R., editor (1993) *Handbook of Chemistry and Physics*, 74th ed. CRC Press, Boca Raton, FL.
40. Pascoe, R. J. and J. P. Foley (2003) Characterization of surfactant and phospholipids vesicles for use as pseudostationary phases in electrokinetic chromatography. *Electrophoresis* **24**, 4227–4240.
41. Lonchin, S., P. L. Luisi, P. Walde and B. H. Robinson (1999) A matrix effect in mixed phospholipids/fatty acid vesicle formation. *J. Phys. Chem. B* **103**, 10910–10916.
42. Walde, P., R. Wick, M. Fresta, A. Mangone and P. L. Luisi (1994) Autopoietic self-reproduction of fatty acid vesicles. *J. Am. Chem. Soc.* **116**, 11649–11654.
43. Chatgilialoglu C., L. Zambonin, A. Altieri, C. Ferreri, Q. G. Mulazzani and L. Landi (2002) Geometrical isomerism of monounsaturated fatty acids. Thiyl radical catalysis and influence of antioxidant vitamins. *Free Radic. Biol. Med.* **33**, 1681–1692.
44. MacDonald, R. C., R. I. MacDonald, B. Ph. M. Menco, K. Takeshita, N. Subbarao and L. Hu (1991) Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta* **1061**, 297–303.